MOLECULAR AND CELLULAR BIOLOGY, Oct. 1985, p. 2642–2646
0270-7306/85/102642-05$02.00/0

Vol. 5, No. 10

Stabilization of a Specific Nuclear mRNA Precursor by Thyroid Hormone

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Received 11 April 1985/Accepted 18 July 1985

The regulation of a thyroid hormone-responsive gene in rats, designated spot 14, was explored. The expression of this gene in liver is rapidly (<10 min) and markedly (>10-fold) altered by the administration of 3,5,3′-triido-L-thyronine (T_3) to hypothyroid rats (P. Narayan, C. W. Liaw, and H. C. Towle, Proc. Natl. Acad. Sci. USA 81:4687–4691, 1984). To investigate the cellular site at which T_3 acts to induce this hepatic mRNA, we made parallel measurements of the relative levels of spot 14 mRNA and nuclear precursor RNA and of the rate of gene transcription after treatments designed to alter the thyroid status of rats. The relative levels of both the mRNA and nuclear precursor were elevated roughly 5- to 6-fold in euthyroid animals and 9- to 12-fold in hyperthyroid animals over those in hypothyroid controls. However, only a small difference of approximately 1.5-fold was detected in the rate of spot 14 gene transcription. After a single injection of T_3 into hypothyroid animals, a small and transient rise in the transcription rate was detected at 30 min. However, the levels of spot 14 mRNA and nuclear precursor RNA increased much more dramatically throughout the first 4 h of treatment. In both cases, changes in the rate of gene transcription were not capable of accounting for the alterations observed in mRNA levels. Thus, the major site of spot 14 gene regulation by T_3 is at a posttranscriptional level. The proportional changes observed in the nuclear precursor and mRNA levels suggest that the site of control is at the level of stability of the nuclear precursor RNA for spot 14 mRNA.

The cellular actions of thyroid hormone are thought to be initiated by the binding of the hormone to a chromatin-associated receptor (12, 24, 27). Subsequently, this interaction is hypothesized to alter the rates of production of specific nuclear RNA. Evidence supporting this hypothesis has come largely from studies which have found alterations in the concentrations of specific mRNA species in target tissues after thyroid hormone treatment. For example, the addition of 3,5,3′-triiodo-L-thyronine (T_3) to cultured rat pituitary tumor cells led to the induction of growth hormone mRNA (18, 29, 30). This induction was recently found to be associated with changes in the rate of growth hormone gene transcription (6, 31, 35). Thus, the thyroid hormone receptor may act in a fashion analogous to that proposed for the steroid hormone receptor: by binding to specific sites in the 5′-upstream regions of target genes and acting as a positive regulator of gene transcription (2).

We have recently focused our attention on the regulation of the production of a specific mRNA in rat liver designated spot 14 (10, 28). This mRNA was first identified by the nature of its translational product (M_r = 17,000; pI ≈ 4.9), the levels of which increase over 10-fold after treatment of hypothyroid animals with T_3. Spot 14 mRNA can also be induced by T_3 in primary cultures of adult rat hepatocytes (16). When a cDNA clone of spot 14 mRNA was used, the induction of mRNA sequences was found to occur with a lag time of less than 20 min (10, 22). The relatively short lag time for hormonal action suggests that the induction of spot 14 mRNA may be a direct consequence of the thyroid hormone-receptor interaction in the hepatocyte. Spot 14 mRNA is encoded by a single gene in the rat haploid genome which contains only one intervening sequence interrupting the gene in the 3′-untranslated region (15). Recently, we demonstrated that the levels of the nuclear precursor RNA for spot 14 mRNA were elevated by 10 min after hormone treatment of hypothyroid rats (22). The shorter lag time observed for the effects of T_3 on the nuclear precursor than on the mature mRNA implies that the primary site of action of T_3 is in the nucleus. To further investigate the molecular site of action of T_3, we estimated the relative rates of spot 14 gene transcription after T_3 treatment of rats and compared these rates to changes in the relative levels of nuclear precursor and mature mRNA in the same animals. On the basis of our results, we suggest that the primary action of T_3 is not on the rate of spot 14 gene transcription but on the stability of the spot 14 mRNA precursor in the nucleus.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats weighing 200 to 250 g were used in all experiments. Animals were rendered hypothyroid by surgical thyroidectomy, followed by the administration of 0.1 mCi of ^{131}I. Alternatively, hypothyroidism was achieved by the addition of 0.025% (wt/vol) methimazole to drinking H_2O for a period of 3 to 4 weeks (3). Hypothyroid animals were used only after the complete cessation of weight gain for a period of 2 consecutive weeks. Results from the two treatments were comparable and were pooled for datum analyses. Chronic hyperthyroidism was induced by the intraperitoneal injection of 20 μg of T_3 per 100 g of body weight per day for 7 days. For time course experiments, hypothyroid animals were inoculated intravenously with a single dose of 50 μg of T_3 per 100 g of body weight. The animals were maintained on a 12-h light–12-h dark cycle with free access to food (Purina rat chow) and H_2O and were killed between 2 and 4 h into the light cycle to minimize the contributions of the diurnal variation of spot 14 mRNA (10).

Spot 14 cDNA and genomic clones. The two spot 14 cDNA clones, pS14-c1 (790 base pairs) and pS14-c2 (900 base

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pairs), were described previously (15, 22). Together, these cDNA clones represent 1,050 nucleotides of the longer spot 14 mRNA species of 1,265 nucleotides (excluding the polyadenylic acid tail). The 650-base-pair cDNA insert probe (probe A in reference 15) used for dot blot and Northern analyses was excised from pS14-c2 by digestion with *Pst*I and was purified by electrophoresis on a 1.5% agarose gel. Strand-specific probes of the spot 14 gene were prepared by subcloning this 650-base-pair fragment into the bacteriophage M13-mp10 vector. Phage containing single-stranded DNA corresponding to the mRNA sequence were designated mS14-c2$^+$, and those containing single-stranded DNA corresponding to the cDNA sequence were designated mS14-c2$^-$. An intron-specific probe was obtained by purifying the 850-base-pair *Eco*RI fragment from the single intervening sequence of the spot 14 gene. This fragment was subcloned into the *Eco*RI site of pBR322 DNA and designated pS14-g5. This fragment does not contain any repetitive DNA element. A ^{32}P-labeled probe of this fragment hybridized only to the 4,750-nucleotide spot 14 nuclear precursor, as determined by electrophoretic analysis of total rat liver nuclear RNA (data not shown).

Measurements of relative spot 14 mRNA and nuclear precursor RNA levels. Livers from treated animals were divided into three portions for analyses of cellular spot 14 mRNA levels, nuclear precursor RNA levels, and rates of spot 14 gene transcription (see below). The isolation of total cellular RNA by a guanidine hydrochloride extraction procedure and the quantitation of relative spot 14 mRNA levels by the RNA dot blot procedure were performed as previously described (22). Quantitation of dot blot analyses was performed by videodensitometry of autoradiograms (17). The extraction of total nuclear RNA and the measurement of the relative levels of spot 14 nuclear precursor RNA were performed as previously described (22). Total nuclear RNA samples were subjected to electrophoresis on 1.8% agarose gels containing 2.2 M formaldehyde (26), followed by transfer to nitrocellulose (33). The relative intensity of the precursor band of 4,750 nucleotides was quantified by scanning autoradiograms densitometrically. For the time course experiment, the relative level of nuclear precursor RNA was quantitated with the intron-specific probe by RNA dot blot hybridization with nuclear RNA. All data are expressed relative to the average value for the hypothyroid animals, which was normalized to 1.

Measurements of the relative rate of spot 14 gene transcription. Rat liver nuclei were isolated by the procedure of Lamers et al. (11), except that the final centrifugation was carried out through a 2.3 M sucrose cushion. Synthesis and extraction of RNA were performed by the procedure of McKnight and Palmiter (20). The reaction mixture contained, in a volume of 0.1 ml, 20 mM Tris hydrochloride (pH 8.0), 5 mM $MgCl_2$, 150 mM KCl, 16% (vol/vol) glycerol, a 0.4 mM concentration each of ATP, CTP, and GTP, 0.1 mCi of [α-^{32}P]UTP (760 Ci/mmol), and 0.6×10^7 to 1.0×10^7 nuclei. Radiolabeled nuclear RNA ($\sim 3 \times 10^6$ to 6×10^6 cpm) was hybridized to a mixture of pS14-c1 and pS14-c2 plasmid DNA (1 μg each). Hybridization reactions also contained, as an internal standard, 2,000 to 4,000 cpm of ^{3}H-labeled cRNA synthesized from the spot 14 cDNA insert (probe B in reference 15). The relative rates of spot 14 gene transcription, expressed as parts per million, were corrected for nonspecific hybridization to a filter containing 2 μg of pBR322 DNA (5 to 10 ppm) and for the efficiency of the hybridization reaction (40 to 60%). Values were not corrected for the length of the cDNA probe (1,050 base pairs) as compared with that of the entire spot 14 gene (4,425 base pairs).

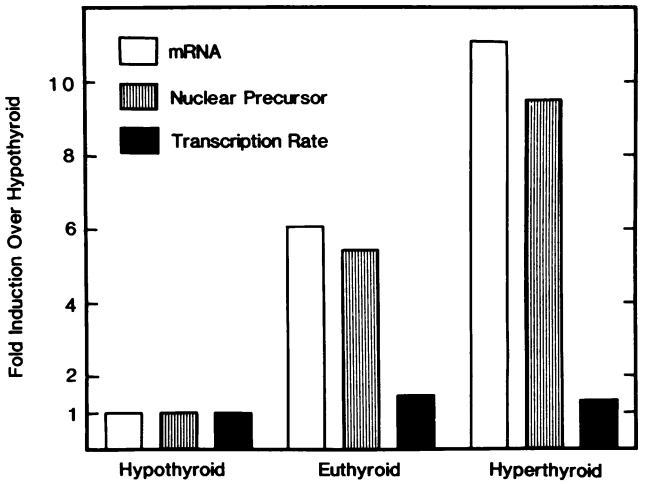

FIG. 1. Comparison of spot 14 mRNA and nuclear precursor RNA levels with rates of gene transcription in various thyroid states. Groups of rats were rendered hypothyroid or hyperthyroid or were untreated (euthyroid). Livers were divided into three portions for analyses of cellular spot 14 mRNA levels, nuclear precursor RNA levels, or rates of spot 14 gene transcription as described in Materials and Methods. Values represent the mean for at least four animals and are expressed as the fold induction over the mean hypothyroid value, which was normalized to 1.

RESULTS

The relative rate of spot 14 gene transcription was measured by the nuclear "run-on" assay (5, 20). Nuclei were isolated from appropriately treated animals and then incubated in vitro in the presence of ^{32}P-labeled ribonucleotides. Under the reaction conditions, RNA chains which were previously initiated in vivo would be elongated, but little RNA initiation or processing would occur. By quantifying the percentage of radioactivity capable of hybridizing to cloned spot 14 cDNA immobilized on nitrocellulose, the relative rate of spot 14 mRNA synthesis can be obtained. This assay has been used extensively to estimate the rate of gene transcription in eucaryotic systems in which direct labeling studies are generally not feasible. For example, the altered production of specific mRNA species mediated by estrogen (1, 19, 20, 32), progesterone (20), glucocorticoid hormone (8), insulin (7), epidermal growth factor (21), cyclic AMP (11), and thyroid hormone (6, 31, 35) has been shown to be due at least in part to alterations in the rates of gene transcription.

Nuclei were isolated from the livers of normal rats or rats which had been treated to render them chronically hypothyroid or hyperthyroid. Parallel measurements were made to determine relative cellular levels of spot 14 mRNA and nuclear precursor and to determine the rate of gene transcription in the same animals (Fig. 1). As reported previously, spot 14 mRNA levels were found to be increased by 5- to 6-fold in euthyroid animals and 9- to 12-fold in hyperthyroid animals relative to hypothyroid animals (10, 22). The relative levels of the nuclear precursor for spot 14 mRNA were elevated roughly in proportion to the changes in mature mRNA. This observation substantiates our earlier conclusion that the primary site of T_3 regulation for spot 14 mRNA is nuclear and precedes the accumulation of the mRNA precursor (22). Measurements of the relative rates of

TABLE 1. Rate of spot 14 gene transcription in hepatic nuclei isolated from rats in different thyroid states

Rat	DNA on filter	Rate of gene transcription[a]
Hypothyroid	pS14-c1 and pS14-c2	104 ± 19
Euthyroid	pS14-c1 and pS14-c2	152 ± 22
Hyperthyroid	pS14-c1 and pS14-c2	135 ± 31
Hypothyroid	pS14-g5 (IVS)[b]	137 ± 6
Hyperthyroid	pS14-g5 (IVS)	112 ± 11

[a] Results are expressed as the mean ppm ± standard error of the mean (n, ≥4). No corrections were made for the relative lengths of the cloned DNA fragments (1,050 base pairs for pS14-c1 and pS14-c2 and 850 base pairs for pS14-g5).

[b] IVS, Intervening sequence.

synthesis of spot 14 mRNA in nuclei isolated from the same animals indicated little differences among the three groups (Table 1). Even in the hypothyroid rats, the hepatic rate of synthesis was substantially high. The rate observed in euthyroid rats was at most 1.5-fold greater than that observed in hypothyroid rats, and no further change was detected in the transition to hyperthyroidism. Thus, the relative changes in the levels of spot 14 mRNA and its nuclear precursor induced by thyroid hormone could not be accounted for by alterations in the rate of gene transcription.

To ensure that the nuclear run-on assay was measuring authentic spot 14 gene transcripts, a number of controls were performed. The amount of ^{32}P-labeled RNA which hybridized to spot 14 cDNA was linearly related to the total input of nuclear RNA over the range of values used (data not shown). No systematic change in the efficiency of hybridization was found in any sample, indicating that endogenous RNA does not compete with labeled RNA for hybridization. RNA synthesized from mouse liver nuclei did not hybridize to a spot 14 cDNA clone from the 3′-untranslated region of the mRNA (Table 2). The production of spot 14 mRNA was inhibited by greater than 90% by the addition of 1 μg of α-amanitin per ml. Moreover, hybridization of ^{32}P-labeled nuclear RNA occurred only to the appropriate template strand of the spot 14 gene. Thus, the run-on assay appears to be capable of detecting spot 14 transcripts synthesized by RNA polymerase II from the appropriate strand of the gene in a quantitative fashion. No change in the rate of spot 14 transcription was detected when hybridization was carried out with cloned DNA from the single intervening sequence of the spot 14 gene (Table 1). Altering the reaction conditions for RNA synthesis to those used by Lamers et al. (11) did not significantly affect the results of the run-on assay (data not shown). We have found that the transcription of the phosphoenolpyruvate carboxykinase gene is reduced by glucose feeding of fasted rats and increased rapidly by subsequent cyclic AMP administration, as reported by Lamers et al. (11). Thus, the conditions used for nuclear isolation and assay appear to be appropriate for detecting bona fide transcriptional changes.

To further investigate the hormonal regulation of the spot 14 gene, we studied the effects of a single injection of a receptor-saturating dose of T_3 in hypothyroid rats (Fig. 2). It has been shown that, after a lag time of approximately 20 min, a nearly linear accumulation of spot 14 mRNA occurs over a period of 4 h (10). Using an intron-specific probe, we found that the levels of the spot 14 mRNA precursor increased dramatically to a maximum at 1 h after T_3 treatment. A 2.7-fold increase ($P \leq 0.01$) in the rate of spot 14 gene transcription during the first 30 min after hormone treatment was observed. A similar increase at 30 min after treatment was found in three other experiments (range, 1.9- to 3.6-fold). However, no further increases in this rate were observed with longer time periods. Instead, the rate of gene transcription actually was reduced towards the basal level with longer treatment times. The early rise in transcription could possibly account in part for the rapid changes observed in the nuclear precursor levels. On the other hand, the continued accumulation of spot 14 mRNA and nuclear precursor at later times is inconsistent with measurements of the transcription rate and indicates that a posttranscriptional process must be the major site of T_3 regulation.

DISCUSSION

We have attempted to determine the primary site at which thyroid hormone acts to control the expression of the spot 14 gene in liver. For this purpose, parallel measurements were made of relative spot 14 mRNA levels and nuclear precursor RNA levels and of the rate of gene transcription after treatments designed to alter plasma T_3 concentrations. The results indicate that T_3 may act at two distinct steps in the production of mRNA. One step is the rate of gene transcription. We found that the rates of spot 14 gene transcription were transiently elevated (~2.7-fold) after a single injection of T_3 into hypothyroid rats. In addition, euthyroid animals had a small, but significant, elevation (~1.5-fold) in the rate of spot 14 gene transcription relative to that in hypothyroid animals. This finding is consistent with recent reports that T_3 acts to transcriptionally activate the growth hormone gene in cultured rat pituitary tumor cells (6, 31, 35). The changes in the transcription rate, however, were relatively minor and do not account for the much greater relative changes observed for the spot 14 mRNA levels. Thus, the major action of T_3 on this gene occurs at a posttranscriptional level. As the levels of the nuclear precursor were altered proportionally to cellular mRNA levels, the posttranscriptional regulation would have to occur at some nuclear step preceding the accumulation of the precursor for spot 14 mRNA. Hence, alterations in RNA splicing, nuclear to cytoplasmic transport, and cytoplasmic mRNA stability can be eliminated as potential major sites of regulation. (This conclusion assumes that an increased accumulation of cytoplasmic mRNA due to stabilization would not "feed back" to alter the levels of the nuclear precursor.) This finding, thus, implies that the stability of the nuclear precursor is altered in response to thyroid hormone. In hypothyroid animals, the gene is transcribed at roughly the same rate as in normal or hyperthyroid

TABLE 2. Analysis of spot 14 RNA synthesis in isolated nuclei

Animal	DNA on filter	Rate of gene transcription[a]
Euthyroid rat	pS14-c1 and pS14-c2	142 ± 12
Euthyroid rat + α-amanitin[b]	pS14-c1 and pS14-c2	10.3
Euthyroid rat	mS14-c2$^-$ (cDNA strand)	140 ± 20
Euthyroid rat	mS14-c2$^+$ (mRNA strand)	0
Euthyroid mouse[c]	pS14-c1	2

[a] Results are expressed as the mean ppm ± standard deviation (n, ≥4). No corrections were made for the relative lengths of the cloned DNA fragments (1,050 base pairs for pS14-c1 and pS14-c2, 630 base pairs each for mS14-c2$^-$ and mS14-c2$^+$, and 720 base pairs for pS14-c1).

[b] α-Amanitin (1 μg/ml) was added to the in vitro incubation mixture to inhibit RNA polymerase II.

[c] pS14-c1 corresponds to sequences from the 3′-untranslated region of spot 14 mRNA which do not cross-hybridize with mouse liver RNA under these conditions.

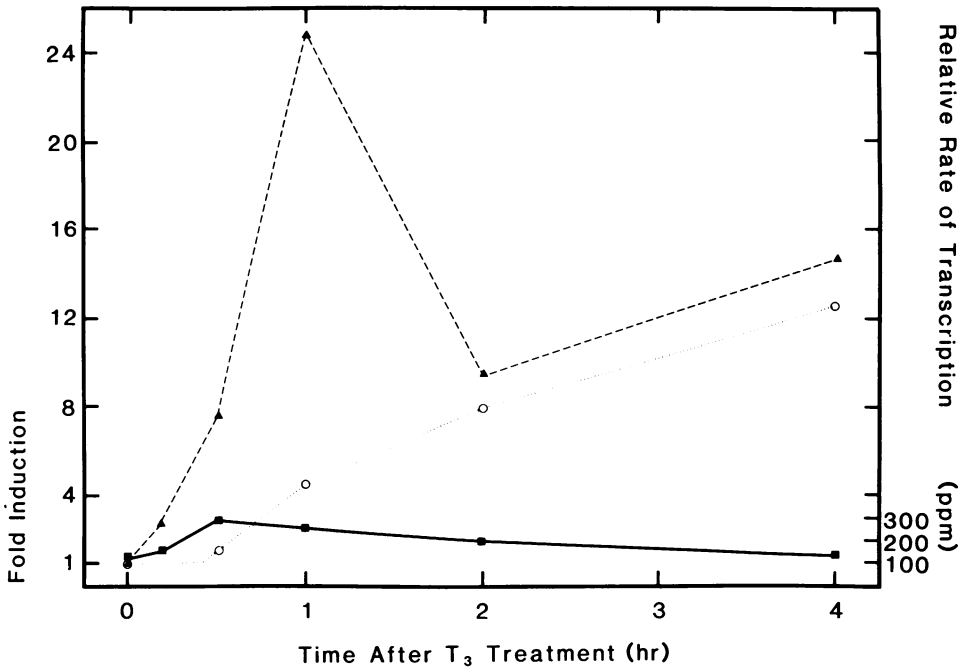

FIG. 2. Time course of the effects of T_3 on spot 14 mRNA and nuclear precursor RNA levels and on the rate of gene transcription. Hypothyroid rats were inoculated in the tail vein with 50 μg of T_3 per 100 g of body weight at time zero. At various times, livers were removed for analysis of relative spot 14 mRNA levels (○) and nuclear precursor RNA levels (▲) and of rates of spot 14 gene transcription (■) as described in Materials and Methods. All values represent the mean for at least four independent samples.

animals, but a large proportion of the precursor is degraded before processing and transport to the cytoplasm take place. In the presence of thyroid hormone, the efficiency with which the primary transcript is processed and transported is increased in a hormone-dependent fashion.

One concern which must be considered is whether the nuclear run-on assay accurately reflects the rate of spot 14 gene transcription. As noted above, this assay has been used in numerous studies in which transcriptional changes have accounted for part or all of the effects of various hormones on specific mRNA levels (1, 6–8, 11, 19–21, 31, 32, 35). In several cases, comparable estimates of gene transcription have been obtained both by using the nuclear run-on assay and by directly pulse-labeling cells to measure transcription (5, 13, 20). All the controls that we ran indicated that the assay was valid. Certainly, the use of strand-specific probes and probes from various parts of the gene (exon versus intron) substantiate the specificity of the hybridization. Nevertheless, the run-on assay is indirect and based on the assumption that initiation is the rate-limiting step in the transcription process. If elongation rather than initiation were rate limiting, then this assay would not be capable of detecting the appropriate changes. Based on available information from both procaryotic and eucaryotic systems, this possibility does not seem likely.

The possibility that regulation may occur through the specific stabilization of a gene transcript ("process versus discard") was suggested many years ago (4). This suggestion was based on the observation that only a portion of all nuclear transcripts are preserved and transported to the cytoplasm in eucaryotic cells (4, 9). However, the regulation of mRNA production at the level of nuclear RNA stability has only been documented in a few studies to date. For example, the androgenic regulation of prostatic binding protein (25) and the regulation of α_1-acid glycoprotein by glucocorticoid hormone in HTC rat hepatoma cells (34) have also been suggested to occur at the level of nuclear RNA stability. The most convincing data for this type of control arise from studies on the regulation of dihydrofolate reductase mRNA during the cell cycle in cultured mouse cells (13, 14). In these studies, direct-labeling kinetics of the products of the amplified dihydrofolate genes were used to verify the nuclear posttranscriptional site of regulation. The stabilization event occurred on the mature (spliced) nuclear mRNA rather than on the nuclear precursor RNA, as observed for spot 14. As additional cases are studied in the appropriate detail, this type of regulation may turn out to be more common than currently appreciated.

The molecular mechanism by which T_3 could act to alter nuclear RNA stability is unclear, particularly in view of evidence suggesting that T_3 acts at a transcriptional level on the growth hormone gene. It is interesting to note that in a recent study, however, the induction of growth hormone gene transcription was also found to be transient and could not account for the continued accumulation of growth hormone mRNA which occurred (23). Thus, other sites of regulation may be utilized to achieve the induction of growth hormone mRNA observed in these pituitary-derived cells. One possible explanation for the effect of T_3 on spot 14 is that it is secondary to the induction of another protein (via a transcriptional route?), which then stabilizes the precursor. If this is true, then the time course of induction of this intermediate would have to be very rapid to account for the kinetics of induction of spot 14 mRNA. On the other hand, T_3 could be acting directly to stabilize the precursor. Perhaps the binding of T_3 to its receptor alters the nuclear localization of the spot 14 gene so that it more readily interacts with components involved in RNA processing. It is also conceivable that the T_3 receptor and the spot 14 nuclear precursor RNA might directly interact with each other.

Although there is little evidence to date to support this notion, further studies to explore this possibility seem warranted. The spot 14 gene is a potentially interesting system for studying not only the complexity of mechanisms by which hormones can act to regulate gene expression but also the processing of gene transcripts in the nucleus.

ACKNOWLEDGMENTS

We thank Julie Engle for technical assistance and Denise McGuire, Steve Seelig, and Jack Oppenheimer for helpful suggestions.

This work was supported by Public Health Service grant AM-26919 from the National Institutes of Health.

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